EUKARYOTIC CELL, Mar. 2006, p. 518–529 Vol. 5, No. 3
1535-9778/06/$08.00+0 doi:10.1128/EC.5.3.518–529.2006

Posttranslational, Translational, and Transcriptional Responses to Nitric Oxide Stress in *Cryptococcus neoformans*: Implications for Virulence†

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Received 28 June 2005/Accepted 26 December 2005

The ability of the fungal pathogen *Cryptococcus neoformans* to evade the mammalian innate immune response and cause disease is partially due to its ability to respond to and survive nitrosative stress. In this study, we use proteomic and genomic approaches to elucidate the response of *C. neoformans* to nitric oxide stress. This nitrosative stress response involves both transcriptional, translational, and posttranslational regulation. Proteomic and genomic analyses reveal changes in expression of stress response genes. In addition, genes involved in cell wall organization, respiration, signal transduction, transport, transcriptional control, and metabolism show altered expression under nitrosative conditions. Posttranslational modifications of transaldolase (Tal1), aconitase (Aco1), and the thiol peroxidase, Tsa1, are regulated during nitrosative stress. One stress-related protein up-regulated in the presence of nitric oxide stress is glutathione reductase (Glr1). To further investigate its functional role during nitrosative stress, a deletion mutant was generated. We show that this *glr1*Δ mutant is sensitive to nitrosative stress and macrophage killing in addition to being avirulent in mice. These studies define the response to nitrosative stress in this important fungal pathogen.

To survive the oxidative and nitrosative attack initiated by phagocytic cells of the host, pathogens must respond appropriately (reviewed in reference 35). This antimicrobial attack is established by two main systems including the inducible nitric oxide synthase pathway and the NADPH oxidase pathway (14). These two pathways generate either reactive nitrogen species (RNS) or reactive oxygen species. In the absence of either of these two pathways, mammalian hosts are more susceptible to both bacterial and fungal infections (18, 41). To cause infection, pathogens must evade the immune system by initiating a response to the stresses encountered.

Previously, transcriptional responses to temperature, osmotic, and hydrogen peroxide stress as well as the stresses encountered in macrophages have been studied in fungi, including *Saccharomyces cerevisiae* and *Candida albicans* (13, 25, 29). A proteomic response to stress has only been determined in *S. cerevisiae* during hydrogen peroxide exposure (17). Proteomic analysis of the nitrosative stress response has not been studied in fungi, though transcriptional responses to RNS have been recently described in *S. cerevisiae*, *C. albicans*, and *Histoplasma capsulatum* (21, 39, 46). Though the response to nitrosative stress has not been studied in *Cryptococcus neoformans*, it has been implicated in both stress resistance and virulence of this fungal pathogen (10, 33, 36). It has been shown that macrophages produce nitric oxide in response to cryptococcal cells (20) and that the anticryptococcal activity of macrophages is mostly dependent on RNS (48). Recently, it was determined that during experimental cryptococcosis, the inducible form of nitric oxide synthase (iNOS) is expressed at increasing levels during infection (30).

RNS are thought to damage cells by oxidizing and nitrating cellular components (24), and specific molecular targets of these harmful species include ribonucleotide reductase and aconitase (37). In this study, we examine the proteomic and transcriptional response of *C. neoformans* to nitric oxide stress. This response consists of transcriptional, translational, and posttranslational regulatory mechanisms. By correlating the proteomic response to RNS with changes in the expression of specific stress response genes, the importance of the stress response to *C. neoformans* survival in macrophages and virulence in mice is demonstrated.

(The data presented are from a dissertation published by T. A. Missall [31a] in partial fulfillment of the requirements for the degree of doctor of philosophy from the Edward A. Doisy Department of Biochemistry and Molecular Biology at Saint Louis University School of Medicine.)

MATERIALS AND METHODS

Fungal strains and media. H99, a well-characterized virulent clinical isolate of *C. neoformans* serotype A, was used as the wild-type strain. *C. neoformans* was grown on rich medium, YPD (1% yeast extract, 2% Bacto peptone, and 2% dextrose), or minimal medium, YNB, pH 4.0 (6.7 g/liter yeast nitrogen base without amino acids plus 20 g/liter dextrose and 25 mM sodium succinate at pH 4). Solid media contained 2% Bacto agar.

Protein lysate preparation. *Cryptococcus neoformans* cells were grown to mid-log phase in YNB, pH 4.0, at 25°C, and then duplicate cultures were treated with 500 μM $NaNO_2$ for 6 h. The cells were collected by centrifugation, washed three times in sterile phosphate-buffered saline (PBS), and resuspended in chilled lysis buffer {40 mM Tris-HCl, pH 9.0, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1× complete protease inhibitor cocktail (Roche), and

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† Supplemental material for this article may be found at http://ec.asm.org/.

1 mM EDTA} at a concentration of 2×10^9 cells/ml. One milliliter of cells and 2.2 g of 0.5 mm zirconium/silica beads were added together in a 2-ml tube, and the cells were disrupted on a Biospec BeadBeater for 30 s at 50,000 rpm, repeated six times, alternated with 2 min on ice. The cell debris was removed by centrifugation (10,000 rpm for 20 min) and syringe filtered (0.45 μm). Dithiothreitol was added to a final concentration of 20 mM. The supernatant was assayed using a Bio-Rad RC-DC protein assay. Typical lysates result in 5 to 7 mg/ml protein. The Amersham 2-D Clean-Up kit was used according to the manufacturer's recommendations to remove nonprotein contaminants. The lysates were then reassayed for protein concentration.

Two-dimensional gel electrophoresis. The isoelectric focusing was done according to the guidelines in the manual provided with the Amersham Pharmacia IPGphor IEF system using a 13-cm Immobiline DryStrip, pH 4 to 7. The strips were then placed on top of a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel in a Hoefer SE 600 vertical electrophoresis apparatus and sealed with 0.5% agarose containing bromophenol blue. Electrophoresis was carried out for 30 min at 15 mA per gel and then at 30 mA per gel with a Tris-glycine-SDS running buffer. After electrophoresis, the gels were stained using the PlusOne protein silver staining kit (Amersham Pharmacia) according to the manufacturer's specifications. Three sets of protein gels were scanned on a densitometer and quantitated using Image Quant.

Excision and trypsinization of protein spots. Gel spots were excised and prepared for proteolytic digestion (16). The gel pieces were dried in a SpeedVac, rehydrated in 1 mM $CaCl_2$ and 50 mM NH_4HCO_3 containing 2 to 4 ng/μl trypsin for protein digestion, and incubated overnight at 37°C. The supernatant was saved, and the remaining peptides were extracted from the gel by three washes in 50% acetonitrile, 5% trifluoroacetic acid. The supernatant and the washes were pooled, and the volume was reduced to 1 μl in a SpeedVac. The peptides were resuspended in 10 μl of 0.2% trifluoroacetic acid, passed through a ZipTip μC_{18} (Millipore) to remove salts, and eluted with matrix (α-cyanno-4-hydroxycinnamic acid; Agilent Technologies).

MALDI-TOF. The molecular masses of the mixtures of trypsinized peptides were determined on a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer. Peptide matrix mixture (0.6 μl) was added to a well on a 96-well MALDI-TOF plate and allowed to air dry. The plate was loaded onto a Voyager-DE Pro Biospectrometry workstation (PerSeptive Biosystems), and mass spectroscopy was done in the reflector mode. Once a spectrum was obtained, it was analyzed with DATA EXPLORER software (version 3.2). The peptide peaks were calibrated using a set of peptides of known molecular mass. A database of *C. neoformans* Twinscan-predicted proteins (August 2003) and TIGR-predicted proteins (October 2003) was used for protein determination. The spectra were compared against this database using the PROTEIN PROSPECTOR software (University of California, San Francisco, CA) with MS-Fit and allowing for 100 ppm (0.01%) error in molecular mass.

RNA extraction and cDNA synthesis. Following the appropriate treatments, 50 ml of *C. neoformans* cells was collected by centrifugation at $1,800 \times g$ for 5 min, washed once with distilled water, and lyophilized overnight. The lyophilized pellet was then vortexed with 3 ml glass beads (1 mm; Biospec, Inc.) and resuspended in 4 ml TRIzol Reagent (Invitrogen). After sitting at room temperature for 5 min, 800 μl chloroform was added and the mixture was shaken for 30 s. This cell lysate was then centrifuged at 4,000 rpm for 10 min, and the supernatant was transferred to a new tube. Two milliliters of isopropanol was added, incubated for 10 min at room temperature, and centrifuged at 4,000 rpm for 10 min. After washing the pellet with 75% ethanol, it was resuspended in water and incubated with DNase I at 37°C for 1 h. The RNA was extracted again with TRIzol and chloroform and precipitated with isopropanol as above. The dried pellet was resuspended in 300 μl RNase-free water (Gibco) and stored at −80°C. All RNA samples were generated in triplicate. First-strand cDNA was made using the First-Strand cDNA synthesis kit for reverse transcription-PCR (Roche).

Microarray construction. Oligonucleotides designed against the TIGR- and Twinscan-predicted protein databases of the serotype D JEC21 strain were synthesized by standard methods by Illumina (San Diego, CA). The oligonucleotides were dissolved at a concentration of 20 μM in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.75 M betaine and were printed in duplicate on Corning Epoxy slides by a locally constructed linear servo arrayer (after the DeRisi model; http://derisilab.ucsf.edu/). Details and availability of the microarray can be found at http://genomeold.wustl.edu/projects/cneoformans/microarray.

Probe labeling. Fluorescence-labeled cDNA was synthesized from 2 μg (each) of total RNA samples using 3DNA capture sequence primers (3DNA Array 900 detection system, Genisphere, Hatfield, PA) and SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) for each probe according to standard protocols. cDNA was concentrated by a Microcon YM-100 filter (Millipore) and used immediately.

Microarray hybridization, scanning, and analysis. A two-step protocol was used for hybridization (3DNA Array 900 detection system, Genisphere, Hatfield, PA). First, oligonucleotide arrays were hybridized to the cDNA probes at 43°C in 2× SDS hybridization buffer and washed in 2× SSC, 0.2% SDS. Fluorescent Cy3- and Cy5-capture dendrimers were combined in hybridization buffer and added to each array. The arrays were incubated and washed as described above.

Slides were scanned immediately after hybridization on a ScanArray Express HT scanner (Perkin Elmer, Boston, MA) to detect Cy3 and Cy5 fluorescence at 543 nm and 633 nm, respectively. Laser power was kept constant for Cy3 and Cy5 scans, and photomultiplier tube (PMT) values were 69 and 58 V, respectively. An additional scan was done for each slide with the PMT set for 55 and 46 V to characterize spots which were saturated at the higher PMT setting. Analysis of images was performed with ScanArray Express software, version 3.0 (Perkin Elmer, Boston, MA). Each spot was defined on a pixel-by-pixel basis, using a modified Mann-Whitney statistical test.

Oligonucleotide elements that received a "present" call (intensity > 200 RFU or local signal-to-background > 2) by the ScanArray software in half of the scans in either the Cy3 or Cy5 were identified, and all others were excluded from the analysis. Since the high PMT data resulted in a larger percentage of "present" spots, only the high PMT data were further considered.

The resulting values were imported into GeneSpring 7 software (Agilent, Redwood City, CA). The data were then handled in the following manner. The local background intensity was subtracted from individual spot intensities. To account for dye swap, the "signal" channel and "control" channel measurements were reversed. The mean signal and control intensities of the on-slide duplicate spots were calculated. A Lowess curve was fit to the log intensity versus log ratio plot. Twenty percent of the data were used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than 10 RFU, then 10 was used instead. Signal-to-Lowess (7) adjusted controlled ratios were calculated. The cross-chip averages were derived from the antilog of the mean of the natural log ratios across all microarray replicates.

Real-time PCR. *C. neoformans* H99 was grown in minimal media at 30°C with shaking overnight. Exponentially growing cells were treated with 250 μM $NaNO_2$ and allowed to grow at 30°C shaking for 2 h. RNA was extracted and first-strand cDNA made as described above. This cDNA was used as template in a real-time PCR using SYBR green PCR reagents (Sigma) according to the manufacturer's recommendations. The primers used for each gene are listed in Table S2 in the supplemental material. The DNA Engine Opticon (MJ Research, Inc.) was used as the fluorescence detector, with the following protocol for the PCR: 35 s at 94°C, 50 s at 53°C, 50 s at 72°C, and a plate reading was repeated for a total of 40 cycles after a hot start of 4 min at 94°C. A melting curve was performed at the end of the reaction to confirm a single product. A series of 10-fold dilutions of the cDNA was used in both the control and the experimental reactions. The induction data were taken from dilutions that came up 3.3 cycles apart, indicating that the reaction was in the linear range. The data were normalized to actin cDNA expression amplified in the set of PCRs.

Generation of *glr1*Δ deletion construct. An overlap PCR gene deletion technology (9) was used to generate the gene-specific deletion cassette of *GLR1* that included a hygromycin cassette (31) and resulted in the deletion of the entire coding region. The two isolates used in these studies were generated from independent transformations.

Transformation of *C. neoformans*. H99 and mutant strains were transformed using biolistic techniques (22, 47). Cells were grown in YPD to late log phase, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6-μm gold beads (Bio-Rad, Richmond, CA) which were coated with DNA of the target construct according to the manufacturer's recommendations. Following the transformation, the cells were incubated at 30°C for 4 h on nonselective media to allow for recovery and then transferred with 0.8 ml sterile PBS to the appropriate selective media. Transformants were observed in 3 to 5 days.

Analysis of transformants. To isolate stable transformants, all transformants were passaged three times on nonselective YPD medium and then tested for resistance to the appropriate selective marker. Only those transformants that grew equally well on the selective media as on nonselective media were used as stable transformants. A three-primer PCR screen was used to prove homologous integration on both the 5′ and 3′ ends of the deletion construct (38). In this manner, homologous recombinants can be distinguished from the wild type. A PCR screen using primers outside the deletion construct will amplify the entire gene region, demonstrating that a single copy of the transforming DNA had been inserted at the desired locus. Southern blots were performed to screen for single

integration in the genome. There were single bands observed on all Southern blots when probed with a selectable marker-specific probe. Both deletion strains generated for this work had a single deletion construct homologously integrated at the appropriate locus and no other insertions in the genome.

Genomic DNA preparation. Genomic DNA was prepared by a modification of the glass bead DNA extraction protocol described by Fujimura and Sakuma (15). *C. neoformans* cells were suspended in a microcentrifuge tube in 500 μl lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, 1% SDS), with 400 mg glass beads (425 to 600 μm; Sigma G-9268). Cells were disrupted by vortexing for 5 min, followed by a 10-min incubation at 70°C. After brief vortexing, 200 μl 5 M potassium acetate and 150 μl 5 M NaCl were added. The tubes were placed on ice for 20 min and centrifuged at 14,000 rpm for 20 min. The supernatant was mixed with 500 μl phenol-chloroform and spun for 2 min at 14,000 rpm. The aqueous phase was then mixed with 450 μl chloroform and spun for 2 min at 14,000 rpm. The DNA was then precipitated by the addition of 200 μl ethanol, washed with 70% ethanol, dried, and resuspended in 50 μl deionized water.

Southern hybridizations. Approximately 10 μg of genomic DNA from each strain was digested with various restriction endonucleases according to the manufacturer's recommendations. Restriction fragments were separated on a 1% agarose gel and transferred to nylon membranes using a Turbo-Blot apparatus (Schleicher & Schuell) and 10× SSC as the transfer buffer. Probes for Southern analysis were prepared by random priming (random priming kit; Roche) using 50 μCi [α- ^{32}P]dCTP (AA0005; Amersham) according to the manufacturer's instructions. The blots were incubated in 10 ml of a 6× SSC, 0.1% SDS, and 5% nonfat dry milk (Carnation) solution for 1 h at 65°C, then probe was added to this solution, and the blots were hybridized at 65°C overnight. The blots were washed twice in 2× SSC, 0.1% SDS at room temperature for 10 min and once for 10 min in 0.2× SSC, 0.1% SDS that had been prewarmed to 65°C.

Oxidative and nitrosative stress plates. Solid minimal media were made with designated amounts of H_2O_2 or $NaNO_2$. *C. neoformans* strains were grown to mid-log phase in YNB, and 10-fold dilutions were made. Five microliters (each) of the undiluted and diluted cultures for each strain was spotted onto the solid minimal media and grown at 30°C for 2 nights.

Macrophage assay. RAW 264.7 macrophages were diluted to 10^5cells/ml in Dulbecco's modified Eagle medium (DMEM). One hundred microliters (10^4) of macrophages was plated into each well of a pretreated microtiter dish. *C. neoformans* cells grown in YNB pH 4 overnight were diluted in DMEM to 10^5cells/ml. The cryptococcal cells were added to the macrophages at a multiplicity of infection of 1 and incubated at 37°C and 5% CO_2 for 24 h. One hundred microliters of 5% SDS was added to each well, and the mixture was incubated at room temperature for 5 min to lyse the macrophages. Serial dilutions were plated on YPD agar and incubated at 30°C for 2 days. Control wells without macrophages were done for each strain to control for growth of cryptococcal cells in DMEM.

Inhalation mouse model. *Cryptococcus neoformans* strains were grown at 30°C with shaking for 2 nights in YPD. The cells were centrifuged, washed in endotoxin-free PBS, and resuspended in endotoxin-free PBS. The cells were counted on a hemocytometer and diluted to 1×10^7 cells/ml. CBA/J female mice (Jackson Laboratories) were anesthetized and allowed to inhale 5×10^5 (50 μl) cells, which were dripped into the nares (8). Mice were weighed before and during the course of infection. Mice were sacrificed by CO_2 asphyxiation once they reached 80% of their original body weight. At this point, the mice showed signs of being morbidly ill, including a ruffled coat, lethargy, a hunched posture, unstable gait, and loss of appetite.

RESULTS

Concentration-dependent nitric oxide toxicity in *C. neoformans*. Nitric oxide is generated by sodium nitrite at pH 4.0 and has been shown to be deleterious to the growth of *C. neoformans* (1). To determine the appropriate concentration of acidified sodium nitrite for revealing a stress response specific to nitric oxide, we analyzed the effects of various concentrations (0 to 750 μM) on the growth of *C. neoformans*. There were no significant effects on viability at 2 h for any of the concentrations tested, but after 6 h, sodium nitrite inhibited growth of *C. neoformans* in a concentration-dependent fashion (Fig. 1). Since 750 μM sodium nitrite was toxic to the cryptococcal cells and 500 μM appeared to be cytostatic, changes in protein expression were examined with 250 μM and 500 μM $NaNO_2$ for 6 h to ensure that the protein changes observed were in response to nitric oxide stress and not due to severe nitric oxide-mediated toxicity. For transcriptional expression changes, a stress of 2 h with 250 μM $NaNO_2$ was used because previous work had shown that changes in the transcriptional profile were apparent at this shorter time point (33, 34).

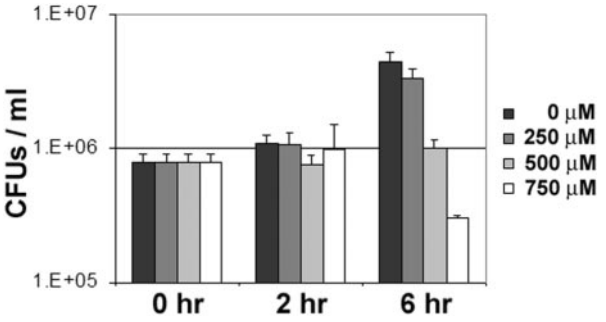
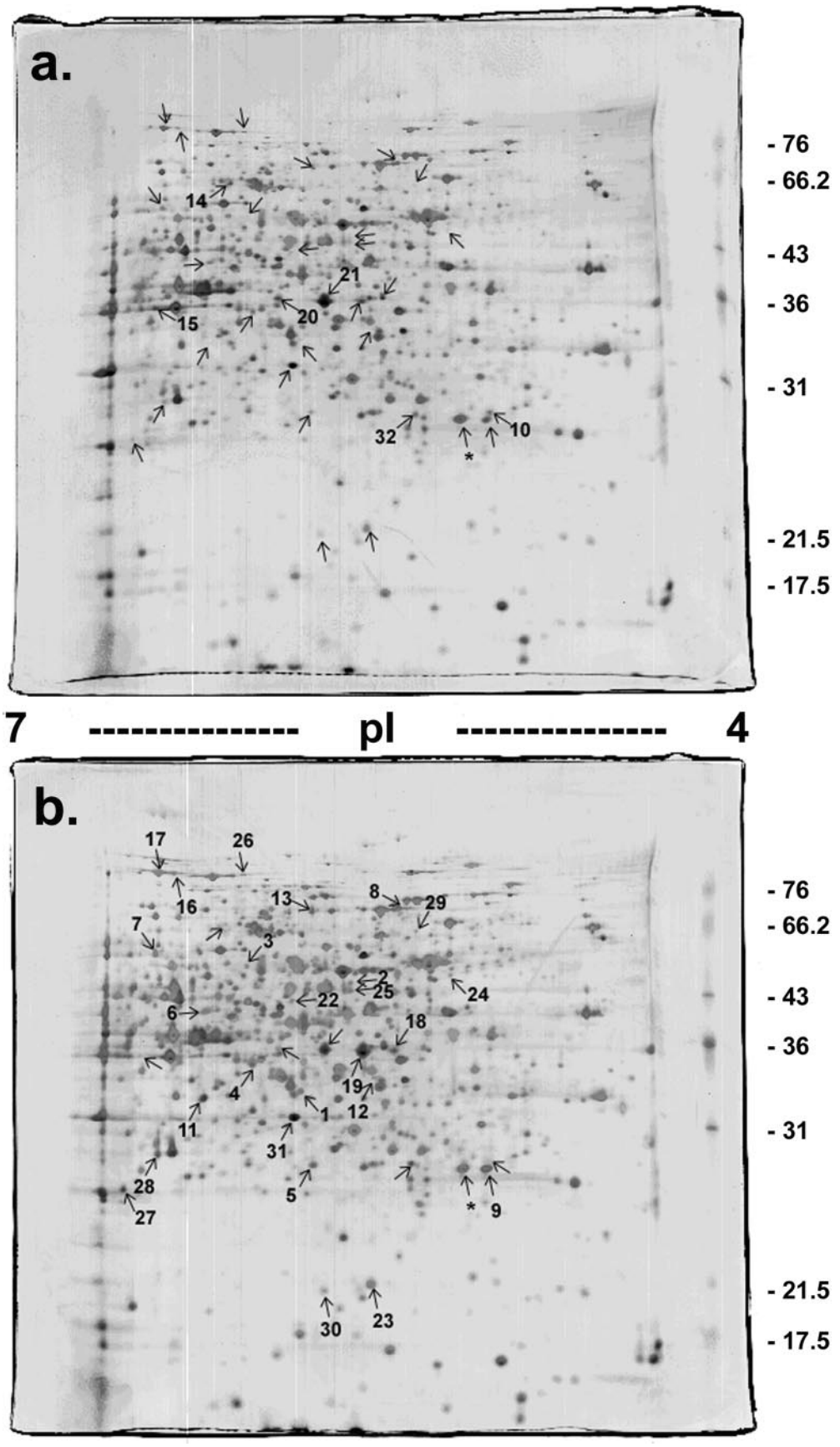


FIG. 1. Survival of H99 in the presence of sodium nitrite at pH 4. Cells were grown for 16 h in YNB pH 4 and treated with various concentrations of sodium nitrite for 2 and 6 h.

Nitric oxide-altered protein expression in *C. neoformans*. To determine the effects of nitrosative stress on protein expression, *C. neoformans* cells were treated for 6 h with 250 μM or 500 μM sodium nitrite at pH 4, and the resulting protein lysates were compared to lysates from untreated cells. To control for effects of low pH or salt, cellular lysates were also prepared from cells that were grown at pH 7 and pH 4 in the absence of sodium nitrite as well as with and without sodium nitrite at pH 7 (data not shown). Comparison of these lysates revealed very minor changes in protein expression (data not shown). When comparing the proteins from cells grown at pH 4.0 with and without sodium nitrite, proteins that displayed a pH- or salt-dependent change were not examined further. Since similar, but magnified, expression changes were observed with 500 μM and 250 μM sodium nitrite stress, the protein spots from the 500 μM lysates were analyzed, since they were more abundant.

Treatment of *C. neoformans* for 6 h with 500 μM sodium nitrite resulted in changes in the steady-state expression levels of over 30 protein spots shown in Fig. 2. Of these 32 protein spots showing altered expression due to nitric oxide, 27 different proteins were identified using peptide mass fingerprinting (Table 1). These included thioredoxin and glutathione antioxidant system proteins as well as putative stress-related oxido-

FIG. 2. Protein expression in response to nitrosative stress. Two-dimensional electrophoretic analysis of H99 untreated (a) and treated with 500 μM sodium nitrite for 6 h (b). Arrows indicate proteins with altered expression which were identified by mass spectrometry, and numbers refer to Table 3. Numbered arrows indicate the gel from which the protein spot was analyzed. Molecular masses are listed in kilodaltons. The asterisk (*) marks the Tsa1 protein spot analyzed in reference 36.


a.
14
21
15
20
32
10
*
- 76
- 66.2
- 43
- 36
- 31
- 21.5
- 17.5
7 ---------- pI ---------- 4
b.
17
26
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11
31
28
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9
27
30
23
- 76
- 66.2
- 43
- 36
- 31
- 21.5
- 17.5

TABLE 1. Proteins identified by mass spectrometry showing altered expression in response to nitric oxide[a]

Role and spot no.	NO effect	Name	Accession no.	Preliminary functional description	Microarray analysis (fold)	Real-time PCR (fold)
Response to stress						
1	New	Fox2	XP_569605	Conserved hypothetical protein; hypothetical oxidoreductase	+	
2	New	Oye2	XP_572706	NADPH dehydrogenase 2 (EC 1.6.99.1), putative	+5	+13.6
3	New	Oye3	XP_566607	Oxidoreductase, putative; NADH-dependent flavin oxidoreductase	+	+8.8
4	↑↑	Oxr3	XP_570275	Oxidoreductase, putative	−1.9	
5	↑↑	Bli3	XP_572673	Conserved hypothetical protein; NcBli3, general stress protein, pyridoxamine 5′-phosphate oxidase	−2.7	
6	↑↑	Oye1	XP_570269	Conserved hypothetical protein; NADH-dependent flavin oxidoreductase		
7	↑	Glr1	XP_570771	Glutathione-disulfide reductase		+3.6
8	↑	Ssc1	XP_567978	Heat shock protein	+2.8	
9	↑↑	Tsa1	XP_571871	Thioredoxin-dependent peroxide reductase		+5.3
*	No change	Tsa1	XP_571871	Thioredoxin-dependent peroxide reductase		+5.3
10	↓↓	Tsa1	XP_571871	Thioredoxin-dependent peroxide reductase		+5.3
Metabolism						
11	New	Oxr1	XP_569236	NADPH isoflavone oxidoreductase, CIP1 protein	+11.6	+27.2
12	New	Cbe1	XP_568314	Carboxylesterase		
13	↑	Gpm1	XP_567598	Phosphoglycerate mutase, putative	+1.6	
14	↓↓	Lat1	XP_570444	Dihydrolipoyllysine-residue acetyltransferase, component of pyruvate dehydrogenase complex		
15	↓↓	Mdh1	XP_572038	Malate dehydrogenase, putative		
16	↑	Aco1	XP_570245	Aconitase	+8.5	
17	↑	Aco1	XP_570245	Aconitase	+8.5	
18	New	Tal1	XP_567910	Transaldolase	+1.1	+5.8
19	↑↑	Tal1	XP_567910	Transaldolase	+1.1	+5.8
20	↓	Tal1	XP_567910	Transaldolase	+1.1	+5.8
21	↓	Tal1	XP_567910	Transaldolase	+1.1	+5.8
Transcription and translation						
22	↑↑	Pbp2	XP_570190	Cytoplasm protein, putative; RNA binding protein		
23	↑	Rps1	XP_569872	Ribosomal protein S19, putative	+1.2	
Signal transduction						
24	↑↑	Hor2	XP_569538	Phosphatase, putative		
Amino acid biosynthesis						
25	New	His5	XP_568883	Histidinol-phosphate transaminase, putative	+3.5	
26	↑	Leu1	XP_566619	3-Isopropylmalate dehydratase	+20.5	
Unknown						
27	↑↑	Ntr1	XP_567659	Conserved hypothetical protein, predicted oxidoreductase related to nitroreductase	+2.9	
28	↑↑	Yhf1	XP_570300	Conserved hypothetical protein, putative NADH-flavin reductase		
29	↑↑	Stg1	XP_571417	Hypothetical protein; serine threonine rich		
30	↑	Mni1	XP_570609	Expressed protein; mannose isomerase related		
31	↑	Msa1	XP_568844	Expressed protein; mucin surface associated	−1.7	
32	↓	Dlh1	XP_572451	Hypothetical protein, dienelactone hydrolase family		

[a] NO effect is the change in protein levels following treatment with $NaNO_2$. "New," a spot that was absent in the untreated control; ↑↑, a consistent increase of greater than twofold; ↑, a consistent increase of less than twofold; ↓, a consistent decrease of less than twofold; ↓↓, a consistent decrease of more than twofold. The transcriptional changes determined by microarray analysis or real-time PCR when determined are aligned. +, an increase in gene expression greater than twofold, with $0.05 < P < 0.06$. Unmarked spots showed inconsistent hybridization among the 12 replicates.

reductases. In addition, proteins with predicted function in amino acid biosynthesis, transcription, translation, signal transduction, and metabolism were identified.

In three separate cases, multiple protein spots were determined by mass spectrometry to be the same protein. These multiple-spot proteins were identified as transaldolase (Tal1), aconitase (Aco1), and the thioredoxin-dependent thiol peroxidase (Tsa1). Six proteins identified with altered expression in response to nitric oxide stress do not share homology to functionally described proteins in other fungi and therefore had been annotated as hypothetical or expressed proteins without a predicted function. This analysis confirms that these predicted proteins are expressed and suggests a potential function. It is possible that these unknown proteins may be unique cryptococcal proteins important for resistance to nitrosative stress. Table 1 shows all the proteins identified by mass spectrometry and the corresponding expression changes under conditions of nitric oxide stress.

Tal1, Aco1, and Tsa1 are posttranslationally modified during nitrosative stress. Multiple protein spots with various isoelectric points for Tal1, Aco1, and Tsa1 were identified. Four distinct spots which run at a molecular mass of 36 kDa with a pI ranging from 5.2 to 5.7 were all identified by mass spectrometry as transaldolase (Fig. 2, spots 18 to 21). One spot (Fig. 2, spot 19) was induced and one spot (Fig. 2, spot 18) was completely new in the nitric oxide-treated gel, while the other two spots (Fig. 2, spots 20 to 21) were reduced in expression in response to nitric oxide. It appears that the modification(s) to Tal1 which occured in response to nitric oxide stress resulted in a decrease in the pI. Similarly, two spots running at about 85 kDa with a pI range from 6.08 to 6.15 were identified as aconitase (Fig. 2, spots 16 and 17). Both of these aconitase protein spots were induced in response to nitric oxide, which correlates well with our microarray analysis (see below). Additional spots were also identified for the thiol peroxidase, Tsa1, which we previously identified using proteomics to be

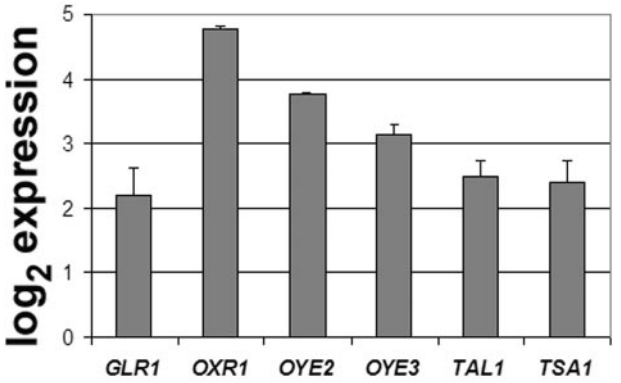

FIG. 3. Gene expression in response to nitric oxide stress. Gene expression was determined by real-time PCR.

induced in response to high-temperature growth (36). The Tsa1 spot we previously described (Fig. 2, spot *) did not change expression in response to nitric oxide, but two other spots showed differential expression during this stress (Fig. 2, spots 9 and 10). Spot 9 was increased by about twofold, while spot 10 was decreased during nitric oxide stress. The various isoelectric points of these three proteins suggests posttranslational modifications associated with nitrosative stress conditions.

Correlation of protein changes with gene expression. Real-time PCR was used to examine whether changes in protein expression correlate with mRNA transcript abundance or are due to altered rates of protein translation, modification, or degradation. Transcriptional changes in response to nitric oxide stress were determined for four genes associated with increased protein expression including *OYE2*, *OYE3*, *GLR1*, and *OXR1* as well as two genes with multiple protein spots, *TAL1* and *TSA1*. All of these genes analyzed were up-regulated during nitrosative stress (Fig. 3). This transcriptional induction correlates with the four proteins induced in response to nitric oxide. In addition, the induction of *TAL1* and *TSA1* indicates both transcriptional and posttranslational regulation mechanisms, since multiple protein changes are observed. These expression data demonstrate the importance of observing protein expression patterns in addition to transcriptional variations and suggest that a large percentage of transcriptional changes correlate with protein expression changes under these conditions.

Microarray analysis of changes in gene expression in response to nitrosative stress. To more completely define the nitrosative stress response in *C. neoformans*, microarray analysis was used. The full-genome microarray (see Materials and Methods) contains 7,737 70-mer oligonucleotides designed against the *C. neoformans* var. *neoformans* (serotype D) strain JEC21. However, these experiments were done using RNA from the *C. neoformans* var. *grubii* (serotype A) strain H99. Although these two strains are similar, many differences occur at the nucleotide level that may interfere with efficient hybridization. During these experiments, 982 spots (see Table S1 in the supplemental material) showed inconsistent hybridization across the spot on a pixel-by-pixel analysis (see Table S1 in the supplemental material). It is not clear if this inconsistency resulted from low levels of expression from these genes, from an inability to hybridize due to high numbers of mismatches, or from uneven spotting of the oligonucleotides. For these experiments, mRNA accumulation was compared in wild-type H99 to H99 treated with 250 μM acidified sodium nitrite for 2 h. Statistical analysis of the 12 biological and technical replicates showed that, of the 7,737 elements, 1,714 had *P* values of less than 0.05, and only these elements were considered for further analysis. Four hundred twenty-one genes showed a nitrosative stress-induced change in expression greater than twofold. Of these genes, 205 were down-regulated between 2- and 27-fold, while the remaining 216 were up-regulated between 2- and 51-fold. These genes included those involved in stress, metabolism, transport, amino acid biosynthesis, cell wall organization and biogenesis, and respiration (Table 2).

Nitric oxide regulated the expression of genes involved in amino acid biosynthesis. Genes whose products are involved in amino acid biosynthesis represented a major class of nitric oxide-responsive genes that are up-regulated (Table 3). These genes are predicted to be involved in the biosynthesis of amino acids including arginine, histidine, leucine, lysine, methionine, and serine. In addition, biosynthetic genes of the branched chain and aromatic amino acids are also induced. Interestingly, glutamate dehydrogenase, involved in glutamate biosynthesis, and a hypothetical protein predicted to be involved in cysteine biosynthesis were down-regulated more than twofold.

To initiate the study of amino acid biosynthesis genes directly to nitric oxide resistance, we tested the two methionine biosynthesis mutants, *met3*Δ and *met6*Δ, and their reconstituted strains (40) for nitric oxide sensitivity. Neither of these mutants showed increased sensitivity to nitric oxide compared to their respective reconstituted strains (data not shown). These two methionine biosynthesis genes have been studied, and both *MET3* and *MET6* were shown to be necessary for

TABLE 2. Summary of gene expression changes in response to nitric oxide stress as determined by microarray analysis[a]

Gene function category	No. of up-regulated genes	No. of down-regulated genes	Total
Amino acid biosynthesis*	20	2	22
Capsule	—	2	2
Cell cycle	2	8	10
Cell wall*	4	8	12
Chromatin maintenance	1	5	6
DNA repair	1	8	9
Iron homeostasis	6	1	7
Carbohydrate metabolism*	9	—	9
Fatty acid metabolism	—	6	6
NAD metabolism	2	—	2
Phospholipid metabolism	—	2	2
Other metabolism	6	5	11
Nucleic acid processing	8	7	15
Protein binding and modification	2	6	8
Respiration*	28	—	28
Response to stress*	10	3	13
Signal transduction	1	2	3
Transcription	2	6	8
Translation	5	1	6
Transport*	20	7	27
Other	14	22	36
Unknown	75	104	179
Total	216	205	421

[a] Gene categories that are discussed in more detail in the text and with a descriptive table are marked with an asterisk; —, absence of data.

TABLE 3. Expression of amino acid biosynthetic genes in response to nitric oxide as determined by microarray analysis

Amino acid	NO effect (fold)[a]	GenBank accession no.	TIGR annotation
Arginine	+2.1	XM_566699	Carbamoyl-phosphate synthase (glutamine-hydrolyzing), putative
	+2.4	XM_569681	Ornithine carbamoyltransferase, putative
	+3.5	XM_570386	Argininosuccinate synthase, putative
Asparagine	+6.8	XM_572287	Asparagine synthase (glutamine-hydrolyzing), putative
Cysteine	−2.1		Hypothetical protein
Glutamate	−2.2	XM_570379	Glutamate dehydrogenase, putative
Histidine	+2.8	XM_567040	Imidazoleglycerol phosphate synthase, putative
	+3.4	XM_570519	Histidinol dehydrogenase, putative
	+3.5	XM_568883	Histidinol-phosphate transaminase, putative
	+9.1	XM_572341	Imidazoleglycerol-phosphate dehydratase, putative
Leucine	+3.0	XM_572334	2-Isopropylmalate synthase, putative
	+20.5	XM_566619	3-Isopropylmalate dehydratase, putative
Lycine	+2.3	XM_571745	Aminoadipate-semialdehyde dehydrogenase (EC 1.2.1.31), putative
	+8.8	XM_570408	Homocitrate synthase, putative
Methionine	+2.9	XM_568037	Homoserine *O*-acetyltransferase, putative
	+5.1	XM_572658	Aspartate kinase, putative
Serine	+4.0	XM_567102	D-3-Phosphoglycerate dehydrogenase 2 (EC 1.1.1.95), putative
Aromatic	+9.7	XM_570499	3-Deoxy-7-phosphoheptulonate synthase, putative
Branched chain	+2.1	XM_572471	Branched-chain amino acid transaminase, putative
	+2.2	XM_568736	Hypothetical protein
	+3.4	XM_566644	Acetolactate synthase, putative
	+3.4	XM_571345	Ketol acid reductoisomerase, putative

[a] NO effect is the change in gene expression following treatment with $NaNO_2$.

virulence (40, 53). Studies of another pathogenic fungus, *C. albicans*, showed the induction of amino acid biosynthetic genes in response to phagocytosis by neutrophils or macrophages (29, 45) but not in response to nitric oxide (21). Since the absence of these methionine biosynthesis genes affected the growth of *C. neoformans*, but not its resistance to nitric oxide, this may suggest that the induction of genes and proteins important for cell nutrition and maintenance is only an indirect or secondary response to nitric oxide stress.

Genes involved in respiration and oxidative metabolism were induced in response to nitric oxide. Genes involved in glycolysis and gluconeogenesis (2- to 27.6-fold), the tricarboxylic acid cycle (2.2- to 9-fold), and mitochondrial respiration (2- to 5.8-fold) were all highly induced during nitric oxide stress (Table 4). Many metabolic enzymes have an iron-sulfur cluster critical to its enzymatic action. Nitric oxide has been shown to interact with iron-sulfur centers, resulting in disassembly and inhibition of enzyme activity (reviewed in reference 12). Specifically, nitric oxide inactivates aconitase and mitochondrial NADH:ubiquinone by binding the iron-sulfur center (4, 5, 44, 51). Mitochondrial electron transport has also been shown to be inhibited by nitric oxide or peroxynitrite by destroying iron-sulfur centers of mitochondrial proteins (43, 51), and we observed the induction of multiple electron transport and mitochondrial transport genes. In addition to the importance of carbohydrate metabolism for cellular respiration, carbohydrate metabolism is critical for NADPH generation and, therefore, redox potential in the cell. These data showing expression changes for genes involved in carbohydrate metabolism and respiration is very reminiscent of expression profiling studies of *C. albicans* in response to internalization by macrophages

TABLE 4. Expression of respiration and carbohydrate metabolism genes during nitric oxide stress as determined by microarray analysis

NO effect (fold)[a]	GenBank accession no.	TIGR annotation
+2.0	XM_571344	Import inner membrane translocase subunit Tim44, mitochondrial precursor, putative
+2.0		Cation-independent mannose-6-phosphate receptor precursor
+2.1	XM_566477	Chaperone regulator, putative
+2.1	XM_570926	Ubiquinol-cytochrome *c* reductase iron-sulfur subunit, mitochondrial precursor (EC 1.10.2.2), putative
+2.1	XM_570793	Mannitol-1-phosphate dehydrogenase, putative
+2.2	XM_571482	Pyruvate carboxylase, putative
+2.2	XM_566692	Succinate dehydrogenase (ubiquinone), putative
+2.3	XM_571507	Cytochrome *c* heme lyase (EC 4.4.1.17) (Cchl), putative
+2.3	XM_570308	NADH dehydrogenase (ubiquinone), putative
+2.4	XM_571714	Electron transporter, transferring electrons within CoQH2-cytochrome *c* reductase complex, putative
+2.4	XM_572394	Hypothetical protein
+2.5	XM_569234	Isocitrate dehydrogenase ($NADP^+$), putative
+2.5	XM_569233	Isocitrate dehydrogenase ($NADP^+$), putative
+2.6	XM_566475	Fructose-bisphosphatase, putative
+2.7	XM_566444	Holocytochrome *c* synthase, putative
+2.7	XM_567499	Mitochondrion protein, putative
+2.7	XM_572905	Kinase, putative
+2.8	XM_572446	NADH-ubiquinone oxidoreductase, putative
+3.1	XM_571637	NADPH-ferrihemoprotein reductase, putative
+3.4	XM_569105	2-Oxoglutarate metabolism-related protein, putative
+3.5	XM_567012	Electron carrier, putative
+3.7	XM_567578	Mitochondrion protein, putative
+3.9	XM_568841	Oxoglutarate dehydrogenase (succinyl transferring), putative
+4.8	XM_569088	Mitochondrial inner membrane protein, putative
+4.9	XM_571601	Hypothetical protein
+5.8	XM_568476	Aconitate hydratase, putative
+5.8	XM_570958	Succinate:fumarate antiporter, putative
+6.2	XM_570152	NADH-ubiquinone oxidoreductase 51-kDa subunit, putative
+6.8	XM_568119	Succinyl-coenzyme A:3-ketoacid-coenzyme A transferase, putative
+7.7	XM_569885	Zinc-binding dehydrogenase, putative
+8.1	XM_572035	Succinate dehydrogenase iron-sulfur subunit, putative
+8.5	XM_570245	Aconitase, putative
+9.0	XM_572035	Succinate dehydrogenase iron-sulfur subunit, putative
+10.7	XM_568803	NADH-ubiquinone oxidoreductase, putative
+27.6	XM_572768	Glyceraldehyde 3-phosphate dehydrogenase, putative

[a] NO effect is the change in gene expression following treatment with $NaNO_2$.

TABLE 5. Expression of cell wall organization and biogenesis genes in response to nitric oxide as determined by microarray analysis

NO effect (fold)[a]	GenBank accession no.	TIGR annotation
−2.7	XM_570869	Cellulase, putative
−2.5	XM_570556	Conserved hypothetical protein
−2.5	XM_568540	Deacetylase, putative
−2.4	XM_570200	Chitin deacetylase, putative
−2.4	XM_570909	Septin, putative
−2.3	XM_570382	Glucosidase, putative
−2.2	XM_568719	1,3-Beta-glucan synthase, putative
−2.2	XM_569656	Protein kinase C, putative
+2.3	XM_567560	Membrane protein, putative
+2.3		Hypothetical protein
+2.5	XM_568689	Chitin synthase, putative
+4.2	XM_572898	Chitinase, putative

[a] NO effect is the change in gene expression following treatment with $NaNO_2$.

which show changes in expression of many metabolic enzymes, including the induction of alternative carbon metabolism (29). In addition, changes in gene expression of carbon metabolism are observed in *C. albicans* and *H. capsulatum* in response to nitric oxide (21, 39). The sensitivity of metabolic and respiratory enzymes to nitric oxide may account for some of the nitric oxide toxicity observed in *C. neoformans*.

Cell wall maintenance and biogenesis gene expression was altered by nitric oxide stress. Our microarray analysis also revealed changes in expression of cell wall organization and biosynthetic genes, a category not identified in our proteomic studies. This could be due to the localization of many cell wall proteins to a cellular fraction that was not included in our proteomic analysis. In response to nitrosative stress, a chitin synthase and endochitinase were induced, possibly suggesting an importance of chitin remodeling to the nitrosative stress resistance. In contrast, there was a general down-regulation of genes involved in cell wall organization and biogenesis, including cellulase, glucosidase, β-(1,3)- and β-(1,6)-glucan synthases, and chitin deacetylase genes (Table 5). These data may suggest that specific cell wall attributes are important to protect the cryptococcal cell from external nitric oxide stress which are distinct from those cell wall properties important for protection against oxidative stress in *C. albicans* and *S. cerevisiae* (6, 50). *C. neoformans* has been recently shown to have high levels of chitin and chitosan in the cell wall, unlike *C. albicans* and *S. cerevisiae* (3). This difference in wall components and their expression in response to stress may reflect a diversity in protective mechanisms among these fungi.

Nitric oxide stress affected expression of transport genes throughout the cell. Many transport-related genes were regulated by nitric oxide stress (Table 6). Upregulation of transporter genes was also observed in *C. albicans* and *H. capsulatum* (21, 39). In *C. neoformans*, genes induced (2.4- to 23.2-fold) included those involved in the transport of amino acids, polyamines, phosphate, nucleic acid, carbohydrates, iron, and the redox cofactor NAD. These data may suggest the importance of nutritional changes and redox potential maintenance in the cell. This transport response to potential nutritional changes correlates well with the induction of amino acid biosynthesis and carbohydrate metabolism genes. Transport-related genes repressed (2- to 5.6-fold) in response to nitric oxide included those involved in vesicle-mediated transport and the transport of fatty acids and calcium.

Various stress response genes are regulated during nitrosative stress. Stress response genes predicted to be important for oxidative and nitrosative stress or heat shock were induced 2.8- to 27.4-fold (Table 7). These included flavohemoglobin denitrosylase, *FHB1*, shown to be important for nitrosative stress and virulence in *C. neoformans* (10), and thioredoxin reductase, *TRR1*, an essential gene shown to be induced during both oxidative and nitrosative stress (34).

Several genes encoding homologs to heat shock genes were also induced. Other stress response genes predicted to be important in osmotic stress or starvation were repressed 2- to 2.7-fold, suggesting the absence of a generalized stress response in *C. neoformans* when treated with nitric oxide.

Glutathione reductase is important to nitric oxide and macrophage resistance and is essential for virulence. This study was done to discover novel proteins that may participate in the stress response of *C. neoformans* to nitric oxide, as these potentially important genes may contribute to the virulence of *C. neoformans*. To test this hypothesis, we generated deletion mutants of glutathione reductase, a gene which has not previously been implicated in nitric oxide stress in any organism but was induced by real-time PCR and proteomic analysis in response to nitric oxide. Two independent *glr1*Δ mutants were isolated, and their sensitivity to nitric oxide and peroxide was evaluated. While mutants deficient in glutathione reductase displayed wild-type resistance to peroxide stress, they were more sensitive to nitric oxide stress than the wild type (Fig. 4a). This is very different from the oxidant sensitivities observed in the *S. cerevisiae* glutathione reductase mutant, which shows sensitivity to peroxide and superoxide stress (19). In addition, it is important to note that the *glr1*Δ mutants show no defects in known virulence factors in vitro, including capsule, melanin production, or high-temperature growth (data not shown). Since nitric oxide resistance has been implicated in survival of *C. neoformans* within macrophages, the ability of the *glr1*Δ mutants to survive in the environment of macrophages was determined. These mutants are more sensitive to killing by RAW 264.7 macrophages compared to the wild type (Fig. 4b). However, treatment of the macrophages with an iNOS inhibitor, L-NMMA, did not reduce the killing of the mutants, suggesting that the sensitivity of the *glr1*Δ mutants was not dependent on nitric oxide (data not shown). As we hypothesized that proteins important to the nitrosative stress resistance of *C. neoformans* may be important to its virulence, we tested the ability of the *glr1*Δ mutants to cause disease in an inhalation model of murine infection. Both independently isolated *glr1*Δ mutants were avirulent in this model, and this suggests that glutathione reductase is essential for virulence in mice (Fig. 4c).

DISCUSSION

Resistance to nitric oxide has been shown to be important to the stress defense and virulence of *C. neoformans* (10, 33, 36). In this study, we characterize the proteomic and transcriptional expression patterns in response to nitric oxide stress. Interestingly, we identify probable posttranslational modifications of three proteins, Tal1, Aco1, and Tsa1, which are altered during

TABLE 6. Expression of various types of transport-associated genes with significant altered expression in response to nitrosative stress as determined by microarray analysis

Transport type	NO effect (fold)[a]	GenBank accession no.	TIGR annotation
Amino acid	+8.1	XM_571087	Amino acid transporter, putative
Calcium	−2.0	XM_570175	Hypothetical protein
Carbohydrate	−2.4	XM_572242	Conserved hypothetical protein
	+2.5	XM_568033	Conserved hypothetical protein
	+2.8	XM_569444	Monosaccharide transporter, putative
	+4.0	XM_572236	Conserved hypothetical protein
	+5.8	XM_572246	Galactose transporter, putative
Fatty acid	−2.0	XM_566978	ATP-binding cassette transporter, putative
Iron	+5.8	XM_571058	Iron ion transport-related protein, putative
Multidrug	+2.4	XM_571586	Multidrug resistance protein Fnx1, putative
	+2.4	XM_570745	Multidrug transporter, putative
	+2.5	XM_570743	Multidrug transporter, putative
	+3.0	XM_570744	Multidrug transporter, putative
Nucleic acid	+2.8	XM_571108	Carrier, putative
	+2.9	XM_572604	Cytosine-purine permease, putative
Phosphate	+23.2	XM_569629	Phosphate transporter, putative
Polyamine	+2.6	XM_571866	Polyamine transport-related protein, putative
	+3.8	XM_567516	Spermine transporter, putative
	+5.0	XM_568464	Conserved hypothetical protein
Redox cofactor NAD	+3.6	XM_570723	Hypothetical protein
Vesicle mediated	−2.4	XM_571601	Actin lateral binding protein, putative
	−2.3	XM_567788	Conserved hypothetical protein
	−2.0	XM_572934	ADP-ribosylation factor, putative
Other	−5.6	XM_567772	Conserved hypothetical protein
	+2.1	XM_568207	Expressed protein
	+2.6		Carrier protein Ymc2, mitochondrial precursor, putative
	+3.9	XM_567049	Hypothetical protein; ABC transporter

[a] NO effect is the change in gene expression following treatment with $NaNO_2$.

TABLE 7. Expression of different types of stress-related genes in response to nitric oxide as determined by microarray analysis

Stress type	NO effect (fold)[a]	GenBank accession no.	TIGR annotation
Heat shock	+2.4	XM_569211	Heat shock protein, putative
	+2.8	XM_567978	Heat shock protein, putative
	+2.8	XM_572184	Chaperone, putative
Osmotic	−2.2	XM_566869	Response to osmotic stress-related protein, putative
	−2.0	XM_568444	Transferase, putative
Oxidative	+2.8	XM_568962	Conserved hypothetical protein
	+3.1	XM_571270	Thioredoxin-disulfide reductase, putative; TRR1
	+5.0	XM_572706	NADPH dehydrogenase 2 (EC 1.6.99.1), putative; OYE2
	+9.9	XM_569844	Response to stress-related protein, putative; flavohemoglobin denitrosylase, FHB1
	+11.6	XM_569236	CIP1 protein, putative
	+27.4	XM_570243	Hypothetical protein; cytochrome *c* peroxidase, CCP1
Starvation	−2.7	XM_567454	Endopeptidase, putative
Other	+13.8	XM_568020	Carbonic anhydrase protein, putative

[a] NO effect is the change in gene expression following treatment with $NaNO_2$.

exposure to nitric oxide. Transaldolase (Tal1) has been shown to be important to oxidative stress resistance in other organisms (23, 49). A proteomics study of the peroxide stimulon in *S. cerevisiae* shows that exposure of yeast cells to hydrogen peroxide results in a resetting of carbohydrate metabolism to redirect carbohydrates to pathways that regenerate NADPH at the expense of glycolysis, such as the pentose phosphate shunt (17). A similar mechanism may be occurring in *C. neoformans* during nitric oxide stress. Transaldolase regulates the levels of NADPH produced through the pentose phosphate shunt, which is necessary for reduction of the main oxidant defense systems in most organisms, including the thioredoxin and glutathione systems. It has been shown in various cell types that the phosphorylation of transaldolase and its activity is correlated with the activity of the antioxidant catalase (26). Though additional studies need to be performed to confirm the type of modification of transaldolase as well as the importance of this modification to stress resistance, preliminary data suggest that neither Tal1 nor Tsa1 are phosphorylated during nitric oxide stress in *C. neoformans* (S. Brown, personal communication).

We have previously described the importance of the thioredoxin-dependent thiol peroxidase, Tsa1, to oxidative and nitrosative stress resistance and virulence of *C. neoformans* (36). Specific posttranslational modifications of the thiol peroxidase, Tsa1, in response to nitric oxide have not been described previously, but we postulate that the thiol residues of this enzyme may be modified, since similar peroxidases have been shown to be sensitive to RNS resulting in S-nitrosylation (11, 27) or oxidation (2, 42) of the thiol residues. The three spots identified as Tsa1 appear to be the result of two different modifications, based on the translocation of the protein spots. Additionally, thiol peroxidase-like enzymes and aconitase have been shown to be nitrosylated in the plant *Arabidopsis thaliana* during nitrosative stress (28).

Aconitase functions in the tricarboxylic acid cycle to isomerize citrate to isocitrate. With this key role in cellular energy production and respiration, aconitase enzyme function in the cell is considered an important marker relative to oxidative metabolism. Aconitase has an iron-sulfur center which is sensitive to inactivation by nitric oxide. Though posttranslational modifications other than nitrosylation have not been described for aconitase, phosphorylation has been shown to play a role in

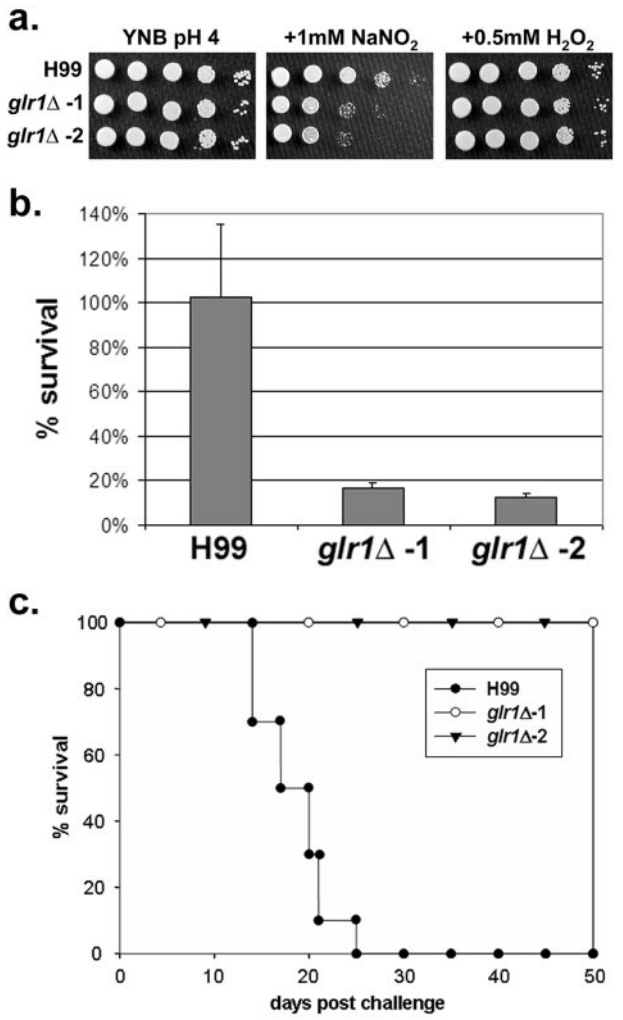

FIG. 4. *glr1*Δ mutant phenotypes. Phenotypes of two independent *glr1*Δ isolates in the presence of oxidative or nitrosative stress (a), in the presence of RAW 264.7 macrophages (b), and in vivo using an inhalation model of murine infection (c).

the stability of iron-sulfur centers (3a). Possibly, aconitase is modified during nitric oxide stress to resist the inhibition of the iron-sulfur center by RNS. Future studies should identify the modifications of these three enzymes implicated in the stress response of *C. neoformans*.

Microarray analysis reveals altered expression of genes related to metabolism, transport, respiration, and amino acid biosynthesis in addition to stress-related genes. Though proteomic analysis reveals similar types of expression changes in response to nitric oxide, the percentage of identified proteins that are stress-related is larger than the percentage of stress-related transcriptional changes observed in the microarray. One of the limitations of gel-electrophoretic proteomic analysis is the ability to visualize the proteins of interest, which is dependent on the abundance of these proteins. We hypothesize that, in general, the abundance of stress-related enzymes is greater than that of metabolic enzymes. Therefore, we attribute the differences in scope between our proteomic and genomic analyses in part to differences in relative abundance of specific subsets of genes or proteins.

Both genomic and proteomic analyses reveal many changes in expression of stress-related enzymes during nitric oxide stress in *C. neoformans*. Interestingly, we observe the specific induction of stress-related proteins involved in oxidative stress and specific down-regulation of stress-related proteins involved in osmotic and starvation stresses. This correlates with gene expression studies performed in *C. albicans* which show specific responses to heat shock, osmotic stress, and oxidative stress rather than a generalized stress response (13). The data presented here also suggest that there are similarities and overlap between oxidative and nitrosative stress responses in *C. neoformans*. These data are supported by recent studies examining the transcriptional response of *C. albicans*, *S. cerevisiae*, and *H. capsulatum* to nitric oxide (21, 39, 46).

We further investigated the importance of glutathione reductase, which is induced both transcriptionally and translationally during nitrosative stress, to the stress response of *C. neoformans* by generating deletion mutants. Glutathione reductase is necessary for reducing the glutathione disulfide, which is formed as a by-product of glutathione peroxidases or glutaredoxins in the presence of reactive oxygen or nitrogen species. The importance of the glutathione peroxidases in *C. neoformans* to the oxidative stress response has recently been described (32), but enzymes of the glutathione system have yet to be shown to be important for the nitrosative stress response in any organism. We find that mutants deficient in Glr1 are sensitive to nitric oxide stress, but not peroxide stress, revealing a specificity between oxidative and nitrosative stress resistance mechanisms in *C. neoformans*. These data reveal the specificity of this antioxidant enzyme to the nitrosative stress response. It is plausible that the absence of the glutathione reductase, Glr1, alters the cellular glutathione levels, which have been shown to influence stress- and non-stress-related pathways necessary for virulence, including sulfur metabolism and transport. In this way, the glutathione system has the potential to regulate a variety of genes, including those important to the virulence of *C. neoformans*. This is supported by the observation that, in addition to the glutathione reductase (this study), the three glutaredoxins also affect the virulence of *C. neoformans* (unpublished data).

Future studies may identify the transcriptional regulator(s) responsible for the induction of nitrosative stress-related genes such as *GLR1*. We also show that Glr1 is important for *C. neoformans* to survive the macrophage attack and is essential for virulence in mice. These data are consistent with the relevance of nitrosative stress resistance studies to virulence in the fungal pathogen *C. neoformans*, although we have not specifically demonstrated that the virulence defect of the *glr1*Δ mutants is caused by nitric oxide sensitivity, as has been shown for flavohemoglobin mutants (*fhb1*Δ) in *C. neoformans* (10). In contrast, a flavohemoglobin mutant in *C. albicans*, *yhb1*Δ, is sensitive to nitric oxide, but the virulence defect in a systemic model of candidiasis is not dependent on the production of nitric oxide by iNOS (21).

Further investigation may elucidate the transcriptional regulatory mechanisms necessary for the induction observed during different stresses in *C. neoformans*, as a unique dual regulation of the thioredoxins has recently been described. A

specificity of the two putative transcription factors, Atf1 and Yap4, was shown to be necessary for induction of the thioredoxin genes in response to oxidative and nitrosative stress, respectively (33). Interestingly, thioredoxin reductase, *TRR1*, which is induced by microarray analysis in response to nitric oxide, has also been shown to be induced during peroxide stress (34). This oxidative stress induction of *TRR1* was shown to be dependent on the putative transcription factor Skn7 (52). We can also show by real-time PCR that *TRR1*, but not *FHB1*, induction during nitrosative stress is dependent on Yap4 (data not shown). This reiterates the unique dual regulation of *C. neoformans* stress resistance genes and that this regulation may vary within and among antioxidant systems. This study, and others aimed at understanding the responses of *C. neoformans* to various stresses, helps define the virulence mechanisms of this fungal pathogen.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Seth Crosby and Michael Heinz at the Washington University School of Medicine Microarray Core Facility and Kristi Stubbert at the Saint Louis University School of Medicine Proteomics Center. We thank J. H. McCusker for providing strains. We also thank the *Cryptococcus* microarray consortium, *C. neoformans* H99 sequencing project, Duke Center for Genome Technology (http://cneo.genetics.duke.edu), the Broad Institute (www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans), the Genome Sequence Centre, BC Cancer Research Centre (www.bcgsc.ca/gc/cryptococcus), the *C. neoformans* serotype D Genome Project, Stanford Genome Technology Center, funded by the NIAID/NIH under cooperative agreement U01 AI47087, and The Institute for Genomic Research, funded by the NIAID/NIH under cooperative agreement U01 AI48594. We thank the *C. neoformans* cDNA sequencing project at University of Oklahoma (http://www.genome.ou.edu/cneo.html), funded by the NIH/NIAID AI147079.

This work was supported by an AHA fellowship to T.A.M. and by NIH/NIAID grants RO1-AI051209 and RO1-AI50184 to J.K.L.

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